TITLE OF THE INVENTION

MUTANT PSEUDOMONAS PUTIDA STRAINS AND METHODS OF USE THEREOF

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

The present invention relates to mutant strains of *Pseudomonas putida* and their use in the detoxification of industrial and/or toxic waste products.

DESCRIPTION OF THE BACKGROUND

Many organic compounds synthesized from petroleum and other chemicals are highly toxic and recalcitrant. Herbicides, insecticides, chlorosubstituted dioxins and PCBs are known biohazardous compounds. Therefore, environmental protection efforts have been focussed on the development of more effective processes for the treatment of these toxic wastes.

Microorganisms used to process these hazardous wastes are likely to be exposed to a wide range of waste concentrations. Thus, this is one of the first problem that must be faced when addressing biological waste treatment issues. Above a certain threshold, toxic chemicals can kill a community of microorganism. Therefore, it is intrinsically important to confer a higher tolerance for waste chemicals, as pointed out by Timmis *et al.* ((1994) *Annu Rev Microbiol* **48**:525-557). Soil bacteria has a wide range of metabolic abilities which make it a useful tool for mineralization of toxic compounds (reviewed by Chaudhry and Chapalamadugu, ((1991) *Microbiol Rev* **55**: 59-79); Esteve-Nunez *et al.*, ((2001) *Microbiol Mol Biol Rev* **65**: 335-352); Kertesz and Wietek, ((2001) *Appl Microbiol Biotechnol* **57**:460-466); Leahy and Colwell, ((1990) *Microbiol Rev* **54**:305-315); Timmis *et al.*, ((1994) *Annu Rev Microbiol* **48**:525-557); Van der Meer *et al.*, ((1992) *Microbiol Rev* **56**:677-694)).

Pseudomonas is a major aerobic microorganism which can mineralize various

organic compounds in environment. The greatest number of strains shown to be tolerant to organic chemicals belong to this genus (Inoue and Horikoshi, ((1989) *Nature*, **388**:264-266); Kim *et al.*, ((1998) *Pseudomonas putida* GM73 *J Bacteriol* **180**:3692-3696); Ramos *et al.*, ((1998) *J Bacteriol* **180**:3323-3329); Weber *et al.*, ((1993) *Appl Environ Microbiol* **59**:3502-3504)). A single species of *Pseudomonas* as well as *Comamonas* is capable of growth on more than 80 organic compounds as a sole source of carbon and energy (reviewed by Palleroni, (1992) Introduction to the family *Pseudomonadaceae*. In *the prokaryotes. A handbook on the biology of bacteria: ecophysiology, isolation, identification, applications. Vol.3* Balows, A., Trüper, H. G., Dworkin, M., Harder, W., and Schleifer, K.-H. (eds.) Springer-Verlag, New York. pp. 3071-3085). The pathways for metabolizing those compounds are highly specific and complex. Most of the enzymes in these catabolic pathways are known to be inducible (Chen *et al.*, (1998) *Res Communs Biochem and Cell & Mol Biol* **2** : 253-274). The discovery of microorganisms capable of tolerating to, or growing on, high concentrations of organic solvents provides a potentially interesting avenue for development of genetically engineered organisms for treating hazardous wastes. Solvent-resistance is inheritable and can be generated by random mutagenesis, and thus appears to be a useful phenotype for waste processing (reviewed by Timmis *et al.*, (1994) *Annu Rev Microbiol* **48**:525-557). Therefore, development of genetically enhanced microorganisms, through the use of genetic engineering, is important for developing better biological waste processing technologies.

In previous work (Hesketh and Ochi, ((1997) *J Antibiot* **50**:532-535); Hosoya *et al.*, ((1998) *Antimirob Agents Chemother* **42**:204-2047); Hu and Ochi, ((2001) *Appl Environ Microbiol* **67**:1885-1892)), we have demonstrated that the secondary metabolism of the genera *Streptomyces* and *Bacillus* (i.e. antibiotic production) could be

activated or enhanced dramatically by introducing a mutation that confers resistance to streptomycin, gentamicin or rifampicin. These mutants have a mutation in the gene which encodes the ribosomal protein S12 (in Str mutants) or the RNA polymerase β-subunit (in Rif mutants) (Hu *et al.*, ((2002) *J Bacteriol* 184: 3984-3991); Inaoka et al., ((2001) *J Bacteriol* **183**:4958-4963); Ochi *et al.*, ((1997) *Mol Gen Genet* **256**:488-498); Shima *et al.*, ((1996) *J Bacterial* **178**:7276-7284); Xu *et al.*, (2002) *Mol Genet Genom,* in press)).

In view of the damaging effects that toxic and industrial wastes pose to the environment and human health there remains a need for improved ways to detoxify these wastes.

SUMMARY OF THE INVENTION

Accordingly, one object of the present invention is to provide methods for increasing the tolerance of *Pseudomonas putida* strains to organic substances, e.g., that are toxic.

Another object of the present invention is to provide novel strains of *Pseudomonas putida* that have increased tolerance to toxic organic compounds.

In addition, by providing such strains, the invention aims to provide improved methods of treating toxic organic compounds, such as those found in industrial waste with these *Pseudomonas putida* strains.

BRIEF DESCRIPTION OF THE DRAWINGS

A more complete appreciation of the invention and many of the attendant advantages thereof will be readily obtained as the same becomes better understood by reference to the following detailed description when considered in connection with the accompanying drawings, wherein:

Figure 1. Growth and consumption of 4HBA by *P. putida* wild-type and mutant strains. The strains were grown to mid-log phase in HBM medium containing 0.3%

4HBA, harvested by centrifugation and resuspended in the same medium to initially give OD_{600}=1.0 and then incubated on a reciprocal shaker. Open symbols show growth, while closed symbols show the concentration of 4HBA. Circle, wild-type KH146-2; square, Str mutant no. 3; triangle, Rif mutant no. 17.

Figure 2. Uptake of 4HBA by the wild-type strain of *P. putida* which was previously grown with either 0.3% 4HBA (●) or 0.3% succinate (■). Uptake of [^{14}C]4HBA was measured as described in Experimental procedures, and the mean values of two separate experiments are shown. Error bars represent the standard deviation.

Figure 3. Uptake of 4HBA by the wild type and mutant strains of *P. putida*. The strains were grown in HBM medium containing 0.3% 4HBA as the sole carbon source, and then uptake of [^{14}C]4HBA was measured as described in Experimental procedures. Error bars represent the standard deviation of the mean of triplicate samples. ●, wild-type KH146-2; ■, Str mutant no. 3; ▲, Rif mutant no. 17; ◇, Str Gen mutant no. 55.

DETAILED DESCRIPTION OF THE INVENTION

The present inventors found that certain Str-, Gen- and/or Rif- mutants derived from *Pseudomonas putida*, such as *Peudomonas putida* KH146-2, which are resistant to streptomycin, gentamicin and/or rifampicin respectively, are tolerant to the aromatic compound 4-hydroxybenzoate (4HBA). In addition to or independent from the Str-, Gen- and/or Rif-mutants, the *Peudomonas putida* strains may also be resistant to other antibiotics as well, for example, fusidic acid, thiostrepton, tetracycline, and others.

The minimum inhibitory concentration (MIC) of 4HBA as the sole carbon source for the wild-type strain was 1%, while the MIC for the mutants was 1.7%. Frequency of 4HBA-tolerant mutants among spontaneous Str-, Gen- and Rif- mutants was 5-15%, 3%, and 3-5%, respectively. These 4HBA-tolerant mutants also tolerated

to a variety of organic chemicals such as 3-hydroxybenzoate, aliphatic and heterocyclic compounds, chlorobenzoates, as well as organic solvents toluene and *m*-xylene.

In view of these findings, the present invention provides methods for increasing the tolerance of *Pseudomonas putida*, such as *Peudomonas putida* KH146-2, to organic substances, e.g., toxic organic substances. The method involves growing a *Pseudomonas putida* strain, which is resistant to one or more antibiotics such as streptomycin, gentamicin, rifampicin, fusidic acid, thiostrepton, tetracycline, and others, in a media using one or more organic substances as the sole carbon source. Obtaining the strains that are resistant to one or more antibiotics can be carried out in a conventional manner which includes growing the strain on a medium containing the one or more antibiotics and isolating those colonies that grow.

Examples of those organic substances include, benzoate and benzoate derivatives, such as 2-hydroxybenzoate, 3-hydroxybenzoate, 4, hydroxybenzoate, cholorobenzoates (including, for example, 2-chlorobenzoate, 3-chlorobenzoate, 4-chlorobenzoate, 2,3-dichlorobenzoate, 2,4-dichlorobenzoate, 4-chlorosalicylate), fluorobenzoate, (including, for example, 2-fluorobenzoate, 3-fluorobenzoate), toluene and toluene derivative, benzene and benzene derivatives, aliphatic compounds, heterocyclic compounds, xylene and xylene derivatives. Other examples include herbicides, insecticides, chlorosubstituted dioxins, and PCBs.

To isolate those *Pseudomonas putida* that have increased tolerance to the organic substance, the strain is cultured in an appropriate medium using the organic substance as the carbon source at increasing concentrations of the organic substance for a period of time that results in the growth of colonies plated on the medium. The initial starting concentration is about 0.8% whereby colonies that grow are transferred to another culture medium containing increasing concentrations by increments of about

1% until a final concentration is about 5%.

As used herein, *Pseudomonas putida* strains with increased tolerance to organic substances means increased relative to the *Pseudomonas putida* strain prior to subjecting it to the method described herein.

The Str mutants had a point mutation in the *rpsL* gene, which produces the ribosomal protein S12.

The Rif mutants were found to have a point mutation in the *rpoB* gene, which encodes the RNA polymerase ß-subunit. Mutation points in Gen mutants still remain unknown. Str-, Gen-, and Rif-phenotypes occurred in spontaneous 4HBA-tolerant mutants which had been selected by successively increasing concentrations (from 0.8% to 5%) of 4HBA. Complementation experiments with one of the Str mutants demonstrated a causal relationship between a *rpsL* mutation (*str-1*) and 4HBA tolerance. Uptake experiments using [^{14}C]4HBA revealed that apparent ability of 4HBA to be taken up by the membrane transport system was reduced 2 to 3-fold in the mutants compared to the wild-type strain, accounting at least partly for the enhanced tolerance to 4HBA.

Thus, *Pseudomonas putida* strains having Arginine in place of Lysine at position 88 in the ribosomal S12 protein and/or mutations in the RNA polymerase β subunit. The sequences of ribosomal protein S12 of *Pseudomonas putida* are known, for example, see GenBank entries NP742615 and AAN66079.1. The RNA polymerase β subunit sequences from *Pseudomonas putida* are also known, for example, see GenBank entries P19175 and 19176.

Preferred examples of *Pseudomonas putida* strains are Str-mutants of *Pseudomonas putida* no. 3, Gen-mutant of *Pseudomonas putida* no. 12, Rif-mutant of *Pseudomonas putida* no. 17; and Str- and Gen-double mutant of *Pseudomonas putida* no.

55, these strains are deposited at the International Patent Organism Depository, National Institute of Advanced Industrial Science and Technology at AIST Tsukuba Central 6, 1-1, Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken 305-8566, Japan and assigned the following accession numbers:

Pseudomonas putida strain no.3—FERM BP-08462

Pseudomonas putida strain no. 12—FERM BP-08463

Pseudomonas putida strain no. 17—FERM BP-08464

Pseudomonas putida strain no. 55—FERM BP-08465

The *Pseudomonas putida* strains can be admixed with one or more suitable carriers individually or together with one or more *Pseudomonas putida* strain and/or other bacterial strains as described herein.

The *Pseudomonas putida* strains described herein can be used to detoxify in whole, or in part, materials containing toxic substances and/or industrial wastes containing such toxic substances. Examples of toxic substances include, benzoate and benzoate derivatives, such as 2-hydroxybenzoate, 3-hydroxybenzoate, 4, hydroxybenzoate, cholorobenzoates (including, for example, 2-chlorobenzoate, 3-chlorobenzoate, 4-chlorobenzoate, 2,3-dichlorobenzoate, 2,4-dichlorobenzoate, 4-chlorosalicylate), fluorobenzoate, (including, for example, 2-fluorobenzoate, 3-fluorobenzoate), toluene and toluene derivative, benzene and benzene derivatives, aliphatic compounds, heterocyclic compounds, xylene and xylene derivatives. Other examples include herbicides, insecticides, chlorosubstituted dioxins, and PCBs.

Sources of toxic and/or industrial waste to be treated are generally known and include, waste receptacles in industrial plants, waste receptacles at an approved or non-approved disposal site, ground water, bodies of water, such as lakes, streams, rivers and the like, soil, household or commercial sources of toxic wastes derived from various

cleaning solutions, solvents and the like.

To treat the toxic and/or industrial waste, the *Pseudomonas* strains described herein are brought in contact with these wastes in amounts and for a time such that a partial or complete degradation of the toxic substances is effectuated. To detoxify waste effectively, it is possible to adjust the concentration of the waste to the upper limit, to which organisms are tolerant for growth. In the case of 4HBA detoxification by *Pseudomonas putida*, the preferable concentration of 4HBA is 2%. Also, two to three times treatment is preferable for complete degradation of the toxic substance.

In so treating these wastes, various combinations of the *Pseudomonas* strains can be employed. Likewise, other bacterial strains in the *Pseudomonocae* family may also be combined, such as, *Commamonas sp.*, e.g., *Commamonas testeroni*. In addition to Pseudomonas and Commamonas, combination with other bacteria such as Rhodococcus may be effective to enhance the ability of detoxification.

A single bacterial strain or mixtures of bacterial strains can be provided as a composition with additional appropriate carriers, such as the media used for growth, water, buffered solutions, etc.

EXAMPLES

Experimental procedures

Bacterial strains, growth conditions, and isolation of mutants

Pseudomonas putida KH146-2 was originally isolated from soil for the capability of growing in the presence of 4-hydroxybenzoate (Chen *et al.*, ((1998) *Res Communs Biochem and Cell & Mol Biol* **2** : 253-274)). HBM medium consists of 50 mM sodium potassium phosphate buffer (pH6.8), 0.1% $(NH_4)_2SO_4$ and Hutner's base. Hutner's

base cotained (per liter) nitrilotriacetic acid, 200 mg; $CaCl_2 \cdot 2H_2O$, 67mg; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 185 μg; $MgSO_4$, 289 mg; EDTA, 2.5 mg; $ZnSO_4 \cdot 7H_2O$, 11 mg; $FeSO_4 \cdot 7H_2O$, 7 mg; $MnSO_4 \cdot H_2O$, 1.5 mg; $CuSO_4 \cdot 5H_2O$, 390 μg; $Co(NO_3)_2 \cdot 6H_2O$, 250 μg, and $Na_2B_4O_7 \cdot 10H_2O$, 180 μg. LB medium contains 1% tryptone, 0.5% yeast extract, and 1% NaCl (adjusted pH 6.8). LBH medium contains 1% tryptone and 0.5% yeast extract in HBM medium. The bacteria were grown at 30°C in a shaker at 200 rpm. For tolerance tests, HBM or LBH agar plates containing serially increasing concentrations of an organic chemical were employed. Routinely, 20 to 40 bacterial strains were spot-transplanted with the aid of tooth pick on an agar plate, followed by incubation at 30°C for 3 days. The lowest concentration in % of an organic chemical that totally inhibits growth was defined as the minimum inhibitory concentration (MIC).

Spontaneous streptomycin-resistant (Str), gentamicin-resistant (Gen) or rifampicin-resistant (Rif) mutants were obtained from colonies that grew within 3 days after wild-type cells were spread on LB agar containing various concentrations of antibiotic.

Isolation of 4HBA tolerant mutants by adaptation

Wild-type strain KH146-2 was adapted, step by step, to grow in HBM medium containing increasing concentrations (from 0.8% to 5%) of 4HBA. In steps using high concentrations (>3%) of 4HBA, adaptation required a longer term incubation (7~10 days) for each step. In certain steps, the 4HBA-adapted mutants were isolated using agar plates (containing the adapted concentrations of 4HBA), on which tolerant mutants formed big size colonies.

Mutation analysis of the rpsL, rplF and rpoB genes

The *rpsL* gene fragment in a Str mutant was obtained by PCR using the mutant genomic DNA as a template and synthetic oligo-nucleotide primers P1 (forward:

5`-CGTAAGACAATCAGTGGAGC-3`-SEQ ID NO:1) and P2 (reverse: 5`-CCGACCTTACTCTTATCGAC-3`-SEQ ID NO:2) which were designed from the sequence of *P. aeruginosa* PA01 (PathoGenesis annotation no. PA268). Similarly, the *rplF* gene in a Gen mutant was obtained using primers P3 (foward: 5'-AAGAACCCCGTCAAGCTCC-3'-SEQ ID NO:3) and P4 (reverse: 5'-TCTTTACGACGGACGACTTC-3'-SEQ ID NO:4). PCRs were performed with Taq polymerase (Takara Ex-Taq) following the manufacturer's instructions. A Perkin-Elmer Cetus thermal cycler was used, and conditions were as follows: 5 min of incubation at 94°C, followed by 30 cycles of 94°C for 0.3 min, 55°C for 0.3 min, 72°C for 0.3 min, and finally at 72°C for 5 min. PCR products were directly sequenced by the dideoxy chain-termination procedure using the Big Dye Terminator Cycle Sequencing kit (Perkin-Elmer Applied Biosystems, Foster City, CA). The sequence data were analyzed with the GENETIX program (Software Development, Tokyo). The partial *rpoB* gene fragment (nt.1524 to 2159) of the Rif mutant was obtained by PCR using a mutant genomic DNA as a template and synthetic oligo-nucleotide primers P5 (forward:5`-TGATCAACGCCAAGCCGGT-3`-SEQ ID NO:5) and P6 (reverse: 5`-TTGCGCTCCATGCCGGTAC-3`-SEQ ID NO:6) which were designed from the *P. putida* sequence (DBGET accession No. X15846). The PCR method and DNA sequencing are the same as those described for the *rpsL* and *rplF* genes.

Cloning of rpsL for complementation tests

To clone the *rpsL* gene, a 0.9-kb DNA region covering whole *rpsL* was amplified from the chromosomal DNA of *P. putida* KH146-2 using KDO DNA polymerase (Toyobo Biochemicals, Osaka). Oligonucleotide primers were designed according to the *rpoC-rpsL-rpsG* sequence of *P. putida* KT2440 (www.tigr.org);

5`-CGTTTTGCCGCTACACGACGTCTTGGCAT-3` (SEQ ID NO:7--nucleotides [nt] -368 to –341 upstream of *rpsL*) and

5`-AAGAGAACGTGGTCGTGGGTCGTCTGATC-3` (SEQ ID NO:8--complementary to nt 107 to 135 downstream of *rpsL*). The amplified DNA fragments were cloned into plasmid pUC118 (Vieira and Messing, (1987) *Methods Enzymol* **153:** 3-11) to confirm the sequence, then excised from the plasmids as a *Bam*HI-*Sph*I fragment using the restriction sites in the multi-cloning sequence of the vector and inserted between the *Bgl*II and *Sph*I sites of the *E. coli-Pseudomonas* shuttle vector pNIT6010 carrying tet^R, a derivative of pNIT6011 (Nishijyo *et al.* (2001) *Mol Microbiol* **40:**917-931). The resultant plasmid pYI433, as well as vector control pNIT6010, was introduced into the Str mutant no. 3 (*str-1*) by conjugation using *E. coli* HB101/pRK2013 as the helper (Comai *et al.* (1983) *Plasmid* **10:**21-30). Fifty μg ml^{-1} of tetracycline was used for selection of transformants.

Assay for the aromatic compounds

Aromatic compounds were assayed using the Hitachi Lachrom HPLC with a cap cell pack C18 UG 1205-5 column (4.6 mm×15 mm; Shiseido, Tokyo) at a flow rate of 1 ml min^{-1} with a mobile phase (CH_3CN : H_2O = 30 : 70, adjusted to pH 2.5 with trifluoroacetic acid). Detection for 4HBA was done using a UV detector set at 230 nm. 4HBA was eluted at 4.25 min.

Measurement of uptake of 4HBA

The method described by Thanassi *et al.* ((1995) *J Bacteriol* **177**:998-1007) was used with some modifications. The bacteria were grown to mid-log phase in HBM medium containing 0.3% 4HBA, washed and resuspended in 0.3% succinate-HBM

medium to give OD_{660} = 0.7. A 5 ml-aliquot of the bacterial suspension in a 100 ml flask was incubated in a shaker bath. A 0.5μCi [carboxyl-^{14}C]4HBA (55 mCi mmol^{-1}, ARC.) with unlabelled 4HBA at increasing concentrations, were added to the 5 ml bacterial suspension and incubation was continued. At the indicated time, a 0.5 ml aliquot of sample was removed and filtered using a nitrocellulose filter (0.45μm pore size, Millipore Corp, Bedford, MA.) which had previously been washed with 2 ml of HBM medium containing 5 mM 4HBA. The sample was applied with a Pipetman, followed by immediate washing with 3 ml of 5 mM 4HBA-HBM medium. The membrane filter was dried, and transferred to a scintillation vial containing Brey`s solution, and radioactivity was measured in scintillation spectrometer. From the rate of uptake and the concentration of 4HBA, apparent Km was determined by reciprocal plotting as described by Beman *et al* ((1983) *J Bacteriol* **156**:1107-1117).

Determination of protein synthesis

Strains were grown to mid-log phase (OD_{660} = 0.7) in 0.3% succinate-HBM medium. A 5 ml aliquot was transferred to each of three 100 ml flasks. [^{14}C(u)]Leucine (300 mCi mmol^{-1}, NEN) and unlabelled leucine were then added to each flask to give a final concentration of 0.1μCi ml^{-1} and 100μM, respectively. At the same time, 4HBA was added to give a final concentration of 0.6% or 1.2% to two of the three flasks. At time zero and at the indicated times thereafter, a 475μl-aliquot was transferred to an Eppendorf tube which contained 25μl of 100% TCA with 100 mM unlabelled leucine. The resulting 5% TCA mixture was left in an ice-water bath for 30 min and then filtered through a nitrocellulose membrane filter. The filter was washed with 5 ml of 5%TCA containing 5 mM leucine (to chase membrane-bound labelled leucine), dried and radioactivity was counted using scintillation spectrometer.

Results

Occurrence of the 4HBA tolerance phenotype among antibiotic-resistant mutants

We first isolated a number of spontaneous streptomycin-resistant (Str), gentamicin-resistant (Gen) and rifampicin-resistant (Rif) mutants of *P. putida* KH146-2. These mutants appeared at a frequency of 10^{-5} - 10^{-6} when KH146-2 cells were spread on LB agar containing various amounts of antibiotics. [Growth of parental strain KH146-2 was completely suppressed with 100μg ml^{-1} of streptomycin, 12μg ml^{-1} of gentamicin, or 10μg ml^{-1} of rifampicin]. Forty mutants resistant to each antibiotic were randomly selected and examined for tolerance to 4-hydroxybenzoic acid (4HBA). 4HBA-tolerant strains, which could grow in the presence of 1% 4HBA, were detected among these antibiotic resistant mutants at a high frequency of 3 to 15% (Table 1). The highest frequency (15%) was detected in the mutants selected with 1000μg ml^{-1} of streptomycin. These 4HBA-tolerant isolates all grew as well as the parent strain on LB agar.

Table 1. Occurrence of 4HBA tolerant mutants among antibiotic resistant isolates of *P. putida*

Concn of antibiotic used for selecting Str, Gen, or Rif mutants (μg ml^{-1})		Frequency of 4HBA tolerant isolates[a]
Streptomycin	500	2/40 (5 %)
Streptomycin	1000	6/40 (15 %)
Gentamicin	20	1/40 (3 %)
Rifampicin	20	2/40 (5 %)
Rifampicin	50	1/40 (3 %)

[a] Forty mutant strains were spot-transplanted onto HBM agar plates containing 1 % 4HBA (as a sole carbon source) and incubated at 30°C. Growth was determined after 6 days incubation.

Tolerance to various organic compounds

Using several strains randomly chosen as representatives of 4HBA-tolerant mutants, we examined the tolerance level to various organic compounds. As summarized in Table 2, the Str mutants and Rif mutants tested all revealed a modest increment of tolerance to 3HBA and to two chlorine-substituted benzoate isomers, 4-chlorobenzoate (4CB) and 2-chlorobenzoate (2CB). However, slight or no increase of tolerance was detected for 2-hydroxybenzoate (2HBA), 3-chlorobenzoate (3CB) and benzoic acid (BA). Tolerance levels to other organic compounds were also examined. We could detect increased tolerance of the Str and Rif mutants to fatty acids (isobutyrate, norvalerate and isovalerate), a heterocyclic compound (isonicotinate), and halogen-substituted benzoate derivatives (4-fluorobenzoate, 2-iodobenzoate, 3-iodobenzoate, 3-bromo-4-hydroxybenzoate, 4-chlorosalicylate and 5-chlorosalicylate) (data not shown).

Table 2. Tolerance (MIC) of *P. putida* mutant strains to benzoate and its derivatives

Strain no.	Concn of antibiotic used for selecting the mutants ($\mu g\ ml^{-1}$)[a]	Benzoate and its derivatives in LBH medium[b]						
		4HBA	3HBA	2HBA	4CB	3CB	2CB	BA
Wild-type		1.4 ± 0.1 (1.1 ± 0.1)[c]	1.4 ± 0.1	0.8 ± 0.1	1.4 ± 0.1	0.9 ± 0.1	1.5 ± 0.2	0.8 ± 0.2
3	1000 (STR)	1.9 ± 0.1 (1.7 ± 0.1)	1.8 ± 0.2	0.9 ± 0.1	1.7 ± 0.1	1.1 ± 0.2	1.9 ± 0.2	1.0 ± 0.1
9	500 (STR)	1.6 ± 0.2 (1.3 ± 0.2)	1.8 ± 0.1	0.8 ± 0.1	1.7 ± 0.1	1.0 ± 0.2	1.7 ± 0.2	0.9 ± 0.1
12	20 (Gen)	1.9 ± 0.3 (1.6 ± 0.2)	1.9 ± 0.2	0.8 ± 0.1	1.6 ± 0.2	1.1 ± 0.2	1.9 ± 0.3	1.0 ± 0.1
14	50 (RIF)	1.8 ± 0.2 (1.4 ± 0.2)	1.9 ± 0.1	0.8 ± 0.1	1.8 ± 0.2	1.1 ± 0.2	1.9 ± 0.2	1.1 ± 0.1
15	20 (RIF)	1.8 ± 0.2 (1.2 ± 0.2)	1.9 ± 0.2	0.8 ± 0.1	1.8 ± 0.1	1.3 ± 0.2	2.0 ± 0.2	1.0 ± 0.1
17	20 (RIF)	1.8 ± 0.2 (1.3 ± 0.1)	1.8 ± 0.3	0.7 ± 0.1	1.7 ± 0.1	1.2 ± 0.1	2.1 ± 0.2	1.1 ± 0.1
18	20 (RIF)	2.0 ± 0.2 (ND[d])	ND	ND	ND	ND	ND	ND
20	20 (RIF)	1.9 ± 0.1 (ND)	ND	ND	ND	ND	ND	ND

[a]STR, GEN, and RIF denote streptomycin, gentamicin, and rifampicin, respectively.

[b]Abbreviations: 2HBA, 3HBA, and 4HBA denote 2-, 3-, and 4- hydroxybenzoate, respectively. BA indicates benzoate. 2CB, 3CB, and 4CB stand for 2-, 3-, and 4-chlorobenzoate, respectively.

[c]The mean value of two to four separate experiments is indicated with the standard deviation. MIC values detected using HBM medium containing 4HBA as a sole carbon source are presented in parentheses.

[d]ND, not determined.

We then examined whether Str and Rif mutants exhibit an increased tolerance to organic solvents such as toluene and xylene. The growth of KH146-2 was inhibited almost completely in LBH medium containing saturated concentrations of *m*-xylene, while Str and Rif mutants grew in this medium. Likewise, growth of KH146-2 was somewhat retarded in 3% MeOH-LBH medium saturated with toluene, but these mutants grew normally indicating correlation of Str and Rif mutations with organic solvent tolerance (data not shown).

Mutational analyses of the mutants

Streptomycin resistance frequently results from a mutation in the *rpsL* gene for the ribosomal protein S12 (Hosoya *et al.*, ((1998) *Antimirob Agents Chemother* **42**:204-2047); Inaoka *et al.*, ((2001) *J Bacteriol* **183**:4958-4963); Shima *et al.*, ((1996) *J Bacterial* **178**:7276-7284)). Rifampicin resistance results from a mutation in the *rpoB* gene (β-subunit of RNA polymerase) (Heep *et al.*, ((1999) *Antimicrob Agents Chemother* **43**:1497-1499); Jin and Gross, ((1988) *J Mol Biol* **202**:45-58); Wichelhaus *et al.*, ((1999) *Antimicrob Agents Chemother* **43**:2813-2816); Hu *et al.*, ((2002) *J Bacteriol* 184: 3984-3991)). We therefore sequenced and compared the *rpsL* gene and *rpoB* gene from the mutants to the wild-type strain. As summarized in Table 3, the eight Str mutants with increased tolerance to 4HBA all contained a mutation in *rpsL* gene at position 263 or 128 resulting in an alteration of Lys-88 to Arg or Lys-43 to Arg, respectively. It is notable that the level of resistance to streptomycin was extremely high (>100 mg ml^{-1}), displaying more than a 1000-fold resistance compared to the wild-type strain (Table 3). Most of the *str* mutants (except for strain no. 9), which were tolerant to 4HBA, had the same mutation which altered Lys-88 to Arg. Although

gentamicin, like streptomycin, is classified as an aminoglycoside antibiotic, the three Gen mutants tested had no mutation in the *rpsL* gene (Table 3). Also, no mutation was detected in the *rplF* gene coding for ribosomal L6 protein (data not shown).

Table 3. Summary of mutations on the *P. putida* genes resulting in tolerance to 4HBA

Strain no.	Relevant genotype	Mutated gene	Nucleotide exchange[a]	Amino acid exchange	Resistance level (μg ml^{-1}) to antibiotic[b]
2	*str-1*	*rpsL*	263A→G	Lys88→Arg	>100,000 (STR)
3	*str-1*	*rpsL*	263A→G	Lys88→Arg	>100,000 (STR)
4	*str-1*	*rpsL*	263A→G	Lys88→Arg	>100,000 (STR)
5	*str-1*	*rpsL*	263A→G	Lys88→Arg	>100,000 (STR)
6	*str-1*	*rpsL*	263A→G	Lys88→Arg	>100,000 (STR)
8	*str-1*	*rpsL*	263A→G	Lys88→Arg	>100,000 (STR)
9	*str-2*	*rpsL*	128A→G	Lys43→Arg	>100,000 (STR)
10	*str-1*	*rpsL*	263A→G	Lys88→Arg	>100,000 (STR)
11	gen	—[c]			50 (GEN)
12	*gen*	—			50 (GEN)
13	*gen*	—			50 (GEN)
14	*rif-1*	*rpoB*	1730T→C	Ile577→Thr	>500 (RIF)
15	*rif-2*	*rpoB*	1549T→C	Ser517→Pro	>500 (RIF)
17	*rif-3*	*rpoB*	1562A→G	Asp521→Gly	500 (RIF)
18	*rif-4*	*rpoB*	1553A→G	Gln518→Arg	500 (RIF)
19	*rif-5*	*rpoB*	1561G→A	Asp521→Asn	500 (RIF)
20	*rif-5*	*rpoB*	1561G→A	Asp521→Asn	500 (RIF)
21	*rif-3*	*rpoB*	1562A→G	Asp521→Gly	500 (RIF)
22	*rif-3*	*rpoB*	1562A→G	Asp521→Gly	500 (RIF)
23	*rif-4*	*rpoB*	1553A→G	Gln518→Arg	500 (RIF)
24	*rif-4*	*rpoB*	1553A→G	Gln518→Arg	500 (RIF)

[a]Numbering originates from the start codon of the open reading frame.
[b]Determined after 1 days of cultivation on LB agar. STR, GEN, and RIF denotes streptomycin, gentamicin, and rifampicin, respectively.
[c]Mutations were not detected within the *rpsL* gene.

In sequencing the *rpoB* gene of the Rif mutants, we focused on a specific region (nt 600 to 1300) corresponding to the Rif domain of *E. coli* (Jin and Gross, (1988) *J Mol Biol* **202**:45-58). The sequencing data obtained for the Rif mutants revealed that all possess a point mutation in the Rif region (Table 3). Thus, the Str mutants and Rif mutants with increased tolerance to 4HBA were found to be tightly linked with the mutations in *rpsL* and *rpoB* genes, respectively. When Str mutants were first selected for resistance to streptomycin, followed by *rpsL* sequencing, these sequenced *rpsL*

mutants (we tested eleven *str-1* mutants and seven *str-2* mutants) all revealed resistance to 4HBA, suggesting a causal relationship between the *str* mutations and 4HBA tolerance.

Complementation experiments for str-1 mutation

We demonstrated further the casual relationship between the *str-1* mutation and 4HBA-tolerance by complementation experiments using a Str mutant no. 3 *(str-1)* (FERM BP-08462) as the host. As expected, strain no. 3, which carries the plasmid pYI433 containing the wild-type *rpsL* gene, exhibited levels of Str about the same as wild-type levels (100-200 $\mu g\ ml^{-1}$). In parallel, the plasmid containing the *rpsL* gene significantly reduced the 4HBA-tolerance of the host when compared with strain no. 3 harbouring a plasmid without *rpsL* (1% versus 1.6%), demonstrating that *str-1* mutation is responsible for the elevated tolerance to 4HBA.

Development of the 4HBA tolerance by sequential adaptation

To verify the significance of Str, Gen, and Rif mutations for the development of tolerance, we next attempted an opposite approach. Namely, we assumed that Str, Gen, and Rif phenotypes might appear in 4HBA-tolerant mutants, which were generated as described in the Experimental procedures. Thus, we first conferred a high level of tolerance by adapting the wild-type cells step by step to increasing concentrations of 4HBA (Table 4). At certain concentrations, 4HBA-tolerant colonies were isolated, and eventually we could obtain mutants that were highly tolerant (up to 5%) to 4HBA. Then, we examined these adapted tolerant cells for resistance to antibiotics. Strikingly, Str, Gen, and Rif traits all appeared at a frequency of 7 to 25% displaying the mutations in rpsL and/or rpoB genes (data not shown), when the screening concentration for

4HBA was 2.9% or 4.3% (Table 4). When 4HBA was up to 5%, the tolerant isolates all acquired the Str and Gen phenotypes. In contrast, the Rif phenotype was no longer detected, and the lack of mutation in the *rpoB* gene was confirmed by DNA sequencing. These Str Gen double mutants all possessed a mutation within *rpsL*, which altered Lys-88 to Arg, but no mutation was detected in the *rplF* gene. These results strongly implicate the intrinsic role of Str, Gen and Rif traits in developing a tolerance to 4HBA. Strain no. 55 (FERM BP-08465) was used for further study as a representative of Str Gen double mutants.

Strain no. 55 as well as other Str Gen double mutants no longer grew on a 5% 4HBA-containing medium (but still showed a tolerance to 3% 4HBA) when strains were grown once on LB medium without 4HBA. This indicates that the cell's physiological changes besides genetic mutations took part in the high-level (5% 4HBA) tolerance.

Table 4. Occurrence of antibiotic resistant traits in 4HBA tolerant mutants from *P. putida*

Concn (%) of 4HBA used for accumulating the 4HBA tolerant mutants[a]	Number of 4HBA tolerant isolates examined	Frequency of isolates resistant to:		
		Streptomycin (500 μg ml^{-1})	Gentamicin (20 μg ml^{-1})	Rifampicin (25 μg ml^{-1})
0.8→1.4→1.8→2.6→2.9	84	6 (7 %)	11 (13 %)	10 (12 %)
2.9→3.4→3.7→4.0→4.3	40	3 (8 %)	10 (25 %)	3 (8 %)
4.3→4.7→5	16	16 (100%)	16 (100%)	0

[a]The wild-type strain *P. putida* KH146-2 was adapted to grow in liquid medium which contains stepwise-increasing (0.8%→5%) concentrations of 4-HBA as described in Experimental procedures. During the course of growth adaptation, tolerant mutants were isolated and tested for antibiotic resistance.

Metabolic activity of 4HBA tolerant mutants

Strains no. 3 (FERM BP-08462) and no. 17 (FERM BP-08464) were chosen for further study as representative Str and Rif mutants, respectively. The growth rate as well as the consumption rate of 4HBA by the mutants were similar to the wild-type strain (Fig. 1). Furthermore, the wild-type and mutant strains both exhibited virtually the same activity for 4HBA 3-hydroxylase, the first enzyme in the 4HBA degradation pathway (data not shown). Also, no difference in degree of suppression of protein synthesis (see Experimental procedures) was detected between the wild-type and mutant strains when grown in the absence or presence (0.6 or 1.2%) of 4HBA (data not shown). These results indicate that the increased 4HBA-tolerance is not a result of the enhancement of metabolic activities or the development of tolerance in protein synthesis machinery (i.e. ribosome).

Uptake of 4HBA by mutant cells

To further investigate the mechanism of tolerance, we conducted experiments for uptake of 4HBA. In preliminary experiments, we found that the uptake of 4HBA is inducible. Cells which had previously grown in media with 4HBA as the sole carbon source consumed [^{14}C]4HBA efficiently, while the cells grown in media with succinate did not consume any [^{14}C]4HBA at least for the initial short term (Fig. 2). Later, [^{14}C]4HBA uptake was detectable, but not efficiently (Fig.2), in agreement with the previous observation that induction of 4HBA 3-hydroxylase by addition of 4HBA requires 20 min (Hosokawa, 1970). Therefore, we first grew cells to mid-log phase in HBM medium containing 0.3% 4HBA and succinate, washed, and resuspended in HBM medium containing only succinate. [^{14}C]4HBA was then added with unlabeled 4HBA at various (50 to 500μM) concentrations. The time course for uptake of [^{14}C]4HBA at

100μM by the wild-type and mutant strains is shown in Fig. 3. The apparent Km value thus obtained was 240μM for wild-type strain and the Str and Rif mutants displayed 2 to 3-fold greater Km values (470μM for the Str mutant, and 620μM for the Rif mutant). Likewise, the Str Gen double mutant no. 55, an isolate with a high level of tolerance, also revealed a reduced uptake for 4HBA (Fig. 3). Similar results were obtained when the uptake experiment was carried out by adjusting the culture medium to pH 8.0 instead of 6.8 (data not shown). Thus, the lowered uptake observed in mutants can be inferred to be a cause, at least in part, for the increased tolerance to 4HBA.

We have developed a new approach for improving the tolerance of bacterial cells to aromatic chemicals by introducing specified mutations or by adapting the cells sequentially to higher concentrations of 4HBA. The efficacy of Str, Gen and/or Rif mutations for activating or enhancing the secondary metabolism (i.e. antibiotic production) has been reported for *Streptomyces*, *Bacillus*, and *Pseudomonas* (Hosoya *et al.*, ((1998) *Antimirob Agents Chemother* **42**:2041-2047); Hu and Ochi, ((2001) *Appl Environ Microbiol* **67**:1885-1892); Hu *et al.*, ((2002) *J Bacteriol* 184: 3984-3991); Xu *et al.*, ((2002) *Mol Genet Genom,* 268:179-189)). Introducing Str mutation into *Pseudomonas pyrrocinia* gives rise to 10-fold increase in the production of the antibiotic pyrrolnitrin (Hosoya *et al.*, ((1998) *Antimirob Agents Chemother* **42**:2041-2047)). Thus, the Str mutation is effective not only for enhancing antibiotic production but for elevating the chemical tolerance in *Pseudomonas*, demonstrating that mutations in a ribosome or RNA polymerase endow the cell with an increased tolerance to aromatic chemicals. Particularly noteworthy is the fact that the high level (5%) 4HBA-tolerant strains isolated after sequential adaptation all possessed a *rpsL* mutation (*str-1*), which altered Lys-88 to Arg, implicating strongly the significance of the *str-1*

mutation for development of high level tolerance. The Gen mutation, though not identified, is also likely to be an essential trait for high level tolerance (Table 4).

The action of streptomycin on bacterial ribosomes has been studied in great detail (reviewed by Wallace et al., ((1979) Streptomycin and related antibiotics. In *Antibiotics V-mechanism of action of antibacterial agents*. F. E. Hahn (ed.), Springer-Verlag, New York, N.Y. pp. 272-303) and Cundliffe, ((1990) Recognition sites for antibiotics within rRNA. In *The ribosome: structure, function, and evolution*. W. E. Hill, A. Dahlberg, R. A. Garrett, P. B. Moore, D. Schlessinger, and J. R. Warner (eds.), American Society for Microbiology, Washington, D.C. pp. 479-490)), and among the numerous effects attributed to this drug, the misreading of the mRNA codons is the best known. Resistance to streptomycin has been shown to result from a limited number of point mutations in the *rpsL* gene, which encodes the ribosomal protein S12. The previously reported amino acid positions in which changes conferred streptomycin resistance in *E. coli, B. subtilis* and *Mycobacterium tuberculosis* are Lys-43, Lys-88, and Pro-91 (Finken *et al.*, ((1993) *Mol Microbiol* **9**:1239-1246.); Honore and Cole, ((1994) *Antimicrob Agents Chemother* **38**:238-242); Inaoka *et al.*, ((2001) *J Bacteriol* **183**:4958-4963); Meier *et al.*, ((1994) *Antimicrob Agents Chemother* **38**:228-233)). In the present study, alterations found in the two sites (Lys-43 and Lys-88) therefore represent previously known positions (Table 3). Gentamicin belongs to the kanamycin class of aminoglycosides but is different from streptomycin when classified on the basis of structural characteristics. Exact mutation points for the Gen mutants remain undetected in our present study, although a certain gentamicin resistance has been shown to result from a mutation in the *rplF* gene, which codes for the ribosomal L6 protein (Buckel *et al.*, ((1977) *Mol Gen Genet* **158**:47-54); Kuhberger *et al.*, ((1979) *Biochemistry* **18**:187-193)). Previous studies dealing with various bacteria have indicated that mutations in the *rpoB* gene, which encodes the β–subunit of RNA polymerase, are responsible for the acquisition of resistance to

rifampicin (Heep *et al.*, ((1999) *Antimicrob Agents Chemother* **43**:1497-1499); Jin and Gross, ((1988) *J Mol Biol* **202**:45-58); Singer *et al.*, ((1993) *J Mol Biol* **231**:1-5); Wichelhaus *et al.*, ((1999) *Antimicrob Agents Chemother* **43**:2813-2816)). Almost all the mutations found are located on a specified conserved region of the *rpoB* gene that can be divided into three clusters (Jin and Gross, 1988). In the present study, all of the Rif mutants examined exhibited a point mutation in clusters I or II (Table 3). It is conceivable that the altered conformational status of the RNA polymerase which may result from a *rif* mutation can give rise to different promoter selectivity (or affinity), directly or indirectly leading to a different level of expression for each gene. Indeed, as demonstrated in *E. coli*, the mutant RNA polymerase may have altered promoter selectivity (Ishihama *et al.*, (1990) Structural and Functional modulation of *Escherichia coli* RNA polymerase. In *Structure and function of nucleic acids and proteins.* Felicia Y., H. Wu and Cheng W. W. (eds.), Raven Press, New York. pp. 153-159.). Although the molecular basis of these *str, gen*, and *rif* mutations for the increased tolerance to aromatic chemicals remains obscure, it is apparent that these mutations ultimately cause physiological changes in the membrane, resulting in reduced permeability as demonstrated using [^{14}C] 4HBA (Fig. 3). This result may explain a part of the increased tolerance.

Gram negative bacteria, including *Pseudomonas*, have a complex membrane system consisting of an outer membrane, an inner membrane, and a periplasmic space (reviewed by Sikkema *et al.*, (1995) *Microbiol Rev* **59**:201-202). Solvent tolerance could be achieved, 1) by reducing the rate of uptake through outer membrane, 2) by increasing the rate of metabolism of the ingested compound, or 3) by increasing the rate of the energy-coupled efflux pump consisting of several kinds of tripartite protein complexes (Aono *et al.*, ((1998) *J Bacteriol* **180**:938-944); Nikaido, ((1996) Multidrug efflux pumps of Gram negative bacteria. *J Bacteriol* **178**:5853-5859); Ramos *et al.*,

((1998) *J Bacteriol* **180**:3323-3329)). It is also possible that 4HBA damage may be exerted at the level of the outer membrane in an effect independent from the metabolism of the compound. Different mechanisms contributing to the solvent tolerance have been found. First, adaptive changes in the composition of the inner and outer membranes have been described. These alterations in the membrane structure suppress the effects of the solvents on membrane stability or limit the rate of diffusion through the membrane. Ramos-González *et al.* ((2001) *Appl Environ Microbiol* **67**:4338-4341) demonstrated that *P. putida* DOT-T1E exposed to a high concentration (1.5%) of 4HBA caused *cis*-to-*trans* isomerization of unsaturated fatty acids in the membrane lipid. In addition to these adaptation mechanisms, there exist efflux systems that actively decrease the amount of the toxic solvent in the cells (Huertas *et al.*, ((2000) *Environ Sci Technol* **34**: 3395-3400); Kim *et al.*, ((1998) *J Bacteriol* **180**:3692-3696); Nishimo and Yamaguchi, ((2001) *J Bacteriol* **183**: 5803-12); reviewed by Isken and de Bont, ((1998) *Extremophiles* **2**:229-238)). Multidrug active efflux systems have recently been recognized as efficient mechanisms for organic solvent resistance in *P. aeruginosa* (Aires *et al.*, ((1999) *Antimicrob Agents Chemother* **43**:2624-2628); Li *et al.*, ((1998) *J Bacteriol* **180**:2987-2991); Mine *et al.*, ((1999) *Antimicrob Agents Chemother* **43**:415-417)), *P. putida* (Kieboom *et al.*, ((1998) *J Bacteriol* **180**:6769-6772); Kieboom and de Bont, ((2001) *Microbiology* **147**:43-51); Rojas *et al.*, ((2001) *J Bacteriol* **183**:3967-3973)), and in *E. coli* (Nishino and Yamaguchi, ((2001) *J Bacteriol* **183**: 5803-12); White *et al.*, ((1997) *J Bacteriol* **179**:6122-6126)). The system contributes also to the energy-dependent efflux of a wide variety of antimicrobial agents, including streptomycin and rifampicin (Kieboom and de Bont, ((2001) *Microbiology* **147**:43-51); Mine *et al.*, ((1999) *Antimicrob Agents Chemother* **43**:415-417); Nishino and Yamaguchi, ((2001) *Mol Microbiol* **40:**917-931)). Therefore, one may suppose that the drug

resistant mutants used here have inadvertently developed additional mutation(s) in an element involved in the efflux system. This possibility, however, can be ruled out, since the *rpsL* mutation was proved to be responsible for the enhanced tolerance to 4HBA (see Results). In this light, the work described by Harwood *et al.* (1994) may be significant. In *P. putida*, the *pcaK* gene product is a transporter ptotein for 4HBA with a deduced amino acid sequence indicative of membership in the major facilitator superfamily of transport proteins. *pcaK* mutants have been shown to accumulate 4HBA at reduced rates (Harwood *et al.*, ((1994) *J Bacteriol* **176**:6479-6488)). Thus, it is possible that our Str, Gen, and Rif mutations repressed (by an entirely unknown mechanism) the expression of *pcaK* gene, resulting in the reduced uptake of 4HBA. Alternatively, since the apparent uptake rate is the sum of the actual uptake (influx) and efflux, it is also possible that these drug resistant mutants have acquired a capacity to overproduce wide-specificity efflux pumps by somehow enhancing the expression of the key genes (such as *marA, mexAB, mexXY, srpABC, ttgABC, ttgDEF* etc.) that play a role in the efflux system. If this is the case, the slower permeation of 4HBA into mutant cells (Fig.3) may be a balance between unaltered influx and increased efflux. Our present findings might help in elucidating the mechanisms of tolerance to toxic organic chemicals particularly its relationship with transcription and translation machinery, although further work is necessary to clarify this mechanism for tolerance. Efficacy of Str and Rif mutations for elevating the cell's tolerance to organic solvents such as toluene and xylene (see Results) implicates the widespread applicability of our method in improving the ability of microorganisms to process chemical wastes.

Obviously, numerous modifications and variations of the present invention are possible in light of the above teachings. It is therefore to be understood that within the scope

of the appended claims, the invention may be practiced otherwise than as specifically described herein.